SHORT COMMUNICATIONS

Analytical ^{13}C NMR: Detection, Quantitation, and Positional Analysis of Butyrate in Butter Oil

ABSTRACT

The amount of butyrate contained in a complex mixture of butter oil triglycerides was 10.3 mole % as determined by natural abundance ^{13}C Fourier transform pulse nuclear magnetic resonance (NMR) spectroscopy. This NMR technique also demonstrated the primary isomeric positioning (>97%) of the butyrl group without the need for altering or fractionating the fat mixture.

INTRODUCTION

The analysis of triglyceride structure has been extensively researched (1). Enzymatic techniques, employing pancreatic lipase, have been a major tool in this work and have resulted in the elucidation of the positional distribution of fatty acids in triglycerides in a large number of natural fats (1), including milk fat (2-5). Nevertheless, when triglycerides, such as those in milk fats, are the substrates under study, the utility of the enzymatic probes has been questioned (6-8). Thus, investigators have taken great pains to develop experimental conditions and alternate techniques in their attempts to satisfactorily interpret data emanating from pancreatic lipase studies of milk fat triglycerides (9-14).

The problems associated with the studies of milk fat triglycerides are obviated when the glycerides are examined directly without prior chemical or enzymatic modification. Nuclear magnetic resonance (NMR) is such a probe and, indeed, studies utilizing proton NMR, in combination with achiral and chiral chemical shift reagents, have been employed to verify the primary versus secondary positioning of fatty acids in synthetic triglycerides which contain saturated chains in combination with either unsaturated (15-17) or branched (18) chain fatty acids and to substantiate the stereospecific positioning of buryric acid in a buryrate rich fraction of hydrogenated milk fat (19).

Our studies with Carbon-13-NMR (C-NMR) confirm the primary positioning of butyric acid in unaltered milk fat triglycerides, and we show that this technique can be easily adapted for the quantitative analysis of butyryl esters by the direct examination of milk fat and other butyryl glycerides.

MATERIALS AND METHODS

Triglycerides

Tributyrin (BBB) was purchased from Eastman Kodak (Rochester, NY) and purified by elution with petroleum ether (30-60 C) through neutral alumina (20). Glyceryl-*rac*-1,2-dipalmitate-3-butyrate (*rac*-PPB), glyceryl-*rac*-1,3-dipalmitate-2-butyrate (*rac*-PBP), and glyceryl-*rac*-1,2-dibutyrate-3-oleate (*rac*-BBO) were synthesized and purified in the laboratory of Dr. R.G. Jensen (University of Connecticut, Storrs, CT) according to established procedures (6,21). Optically clear butter oil was obtained by churning fresh, pasteurized cream, melting the butter at 42 C, and storing overnight at 4 C. The butter was remelted at 63 C, and the buttermilk was removed. The fat phase was centrifuged at 5000 rpm, the oil was removed and filtered through glass wool.

^{13}C-NMR

^{13}C-NMR spectra were taken on a Bruker WH-90 Fourier transform NMR spectrometer, operating at 22.63 MHz with proton noise decoupling, and with a Varian CFT-20 operating at 20.0 MHz. The alkyl region of the spectrum was obtained at a 2000 Hz sweep width and displayed at a sweep width of 500 Hz. Pulses of 90° were used with 1000-2000 accumulations. The spectra were obtained in 0.5-1 M solutions of $CDCl_3$ with all chemical shifts relative to internal tetramethylsilane (TMS). The accuracy of the reported δ-values are within ± 0.02 ppm. Repetition rates of 10 sec were utilized to assure quantitative peak area relationships (22). Measured NOE values for all carbon resonances utilized for quantitative composition evaluations were 2.9 ± 0.15.

RESULTS AND DISCUSSION

During our ^{13}C-NMR studies of the *cis/trans* ratio of various fats and oils (22), we observed an unusual aliphatic chemical shift pattern which was exclusive to butter fat. Based on cal-

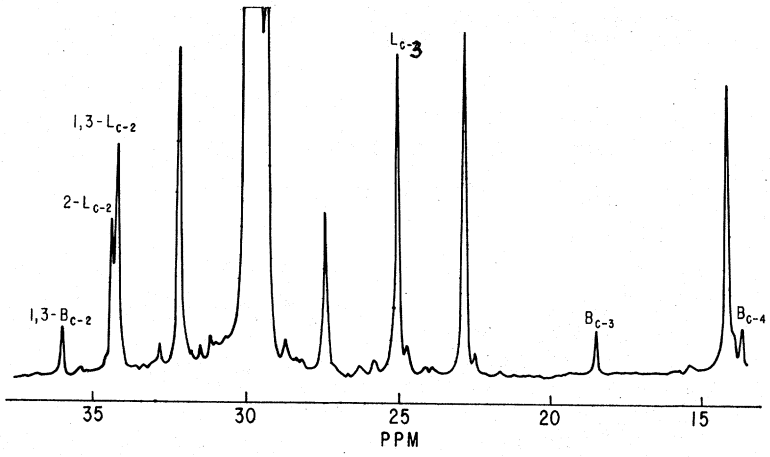

FIG. 1. 22.63 MHz ^{13}C spectrum of butter oil (0.5 g in 1.3 ml of $CDCl_3$). Sweep width of displayed spectrum is 500 Hz. The letter (B) identifies shifts due to butyrate and (L) those due to long chain species.

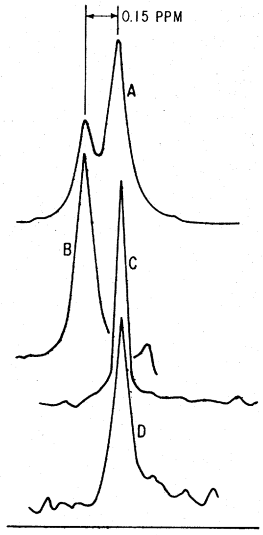

FIG. 2. Plot expansion of butyrate C-2 resonance in (A) BBB, (B) *rac*-PBP, (C) *rac*-PPB, and (D) natural butter oil.

TABLE I

^{13}C Resonance Positions of C-2 in Butyryl and Long Chain Mixed Triglycerides[a]

	δC-2 butyryl		δC-2 long chain	
Triglycerides	1,3	2	1,3	2
Butter oil	35.94	---	34.07	34.24
BBO	35.96	36.14	34.09	---
PPB	35.98	---	34.14	34.30
PBP	---	36.14	34.11	---
BBB	35.96	36.12	---	---

[a]All chemical shifts are reported relative to internal (TMS) in $CDCl_3$.

culated shielding constants (23), the single isolated carbon resonances observed at δ13.63, 18.40, and 35.94 were assigned to carbons 4, 3, and 2 of a butyl ester chain, respectively. The ^{13}C spectrum of butter oil and the position of the three butyrate carbon resonances and other relevant shifts are shown in Figure 1.

By relating the area of the C-3 resonance at δ18.40 to the C-3 resonance area centered at δ24.97, representing all other fatty acid chains, we obtained directly the mole % butyrate content of the unmodified mixture as follows:

$$\text{mole \%, butyrate} = 100 \times \frac{\text{C-3 of butyrate}}{\text{C-3 of butyrate + C-3 of long chains}}$$

Analysis of a freshly prepared butter oil by this procedure showed 10.3 ± 0.3 mole % butyrate content while the value obtained by gas liquid chromatography (GLC) analysis of the derived butyrate (24) was 9.8 mole % ± 0.3%.

On close inspection of the C-2 resonance associated with the butyrate moieties, we observed that, unlike the long chain counterparts, this shift was characterized by a single peak position, (δ35.94). In the long chain esters, however, two C-2 resonances at δ34.07 (area 2) and δ34.24 (area 1) were observed, reflecting esterification at the 1,3- and 2-positions of glycerol, respectively (25). From these observations, it seemed reasonable to conclude that butyrate was restricted primarily to a single isomeric position of glycerol, i.e., 1,3- or 2- in butter. To verify this conclusion and make the correct positional assignment, we examined the ^{13}C spectra of several model compounds including those whose structures corresponded to

the possible compositions we were studying in the natural system. Comparison of the C-2 resonance position as observed in butter oil, *rac*-PPB, *rac*-PBP, *rac*-BBO, and BBB directly confirmed the predominance of butyrate in the primary positions of the butter oil triglycerides (Fig. 2). The limits of detection of any secondary butyryl esters of glycerol in butter were established through a study of mixtures of known composition to be no less than 3% of all butyrate containing triglycerides. In addition to butyrate, the isomeric composition of acetate and propionate (23) triglycerides can also be ascertained in the presence of longer chain esters. However, hexanoate and those of higher molecular weight cannot, due to their common C-2 resonance position. The C-2 shift positions of various butyrate containing triglycerides are listed in Table I.

In previous high field (220 MHz) proton NMR studies (19), stereospecificity, i.e., *sn*-3 positioning of butyrate chains, was ascertained through pseudo contact shift methods following fat fractionation and sample doping with synthetic optical isomers. However, these results were not entirely unambiguous since the pure *sn*-2 isomer was not examined.

Our present report confirms the findings of previous workers (2-5,19) and removes any ambiguities concerning the isomeric positioning of butyric acid in milk fat triglycerides, since no fractionation or alteration of the triglyceride occurred. It also points the way to the further exploitation of ^{13}C-NMR for direct routine quantitative analysis of a variety of unaltered lipid derived materials. A more detailed report concerning the quantitative aspects of this procedure as it pertains to the limitations of detection of butyrate, hexanoate, and free fatty acids will be the subject of a future publication.